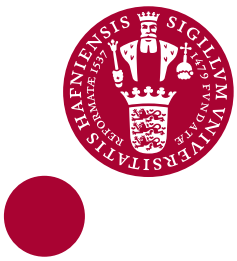

C-Reactive Protein Levels at Diagnosis of Acute Graft-versus-Host Disease Predict Steroid-Refractory Disease, Treatment-Related Mortality, and Overall Survival after Allogeneic Hematopoietic Stem Cell Transplantation

Minculescu, Lia; Kornblit, Brian Thomas; Friis, Lone Smidstrups; Schiødt, Ida; Petersen, Soeren Lykke; Andersen, Niels Smedegaard; Sengeloev, Henrik

Published in:
Biology of Blood and Marrow Transplantation

DOI:
10.1016/j.bbmt.2017.10.025

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Biol Blood Marrow Transplant 24 (2018) 600–607

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

ASBMT American Society for Blood and Marrow Transplantation

C-Reactive Protein Levels at Diagnosis of Acute Graft-versus-Host Disease Predict Steroid-Refractory Disease, Treatment-Related Mortality, and Overall Survival after Allogeneic Hematopoietic Stem Cell Transplantation

Lia Minculescu [1,*], Brian Thomas Kornblit [2], Lone Smidstrups Friis [2], Ida Schiødt [2], Soeren Lykke Petersen [2], Niels Smedegaard Andersen [2], Henrik Sengeloev [2]

[1] *Department of Clinical Immunology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*
[2] *Hematopoietic Stem Cell Transplant Unit, Department of Hematology L, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*

Article history:
Received 30 June 2017
Accepted 15 October 2017

Key Words:
Allogeneic hematopoietic stem cell transplantation
Graft-versus-host disease
Inflammation
C-reactive protein

A B S T R A C T

Acute graft-versus-host disease (aGVHD) remains a cause of excessive morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). Primary treatment consists of high-dose corticosteroids, but a small group of patients develop steroid-refractory disease, and their prognosis is especially poor. There is experimental evidence that coexisting inflammation aggravates aGVHD. Because C-reactive protein (CRP) is a systemic inflammatory marker, we aimed to investigate whether plasma CRP concentrations at the diagnosis of aGVHD can predict the risk of failing first-line therapy and developing steroid-refractory disease. We retrospectively studied 461 patients who underwent HSCT between 2010 and 2015. aGVHD grade II-IV was diagnosed in 148 patients (32%). CRP level and total white blood cell, lymphocyte, and neutrophil counts were available for all patients at the time of aGVHD diagnosis. According to local protocol, patients with failed response to high-dose steroid therapy (2 mg/kg) were treated with the TNF-α inhibitor infliximab and categorized as having steroid-refractory disease. Of 148 patients with grade II-IV aGVHD, 28 (19%) developed steroid-refractory disease. In these patients, plasma CRP concentration at diagnosis ranged between <1 and 253 mg/L. CRP levels were significantly higher in patients who developed steroid-refractory disease compared with those who responded to high-dose corticosteroid therapy (odds ratio, 1.50; 95% confidence interval, 1.18-1.93; $P = .001$). This translated into significantly increased transplantation-related mortality and decreased overall survival in the patients with high CRP levels. Total white blood cell, lymphocyte, and neutrophil counts were not associated with steroid resistance in the patients with aGVHD. These results suggest that CRP level at diagnosis is a valid predictor of the development of steroid-refractory disease in patients who develop grade II-IV aGVHD after HSCT.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potential cure for a variety of malignant and nonmalignant diseases. However, the alloreactivity required for the graft-versus-leukemia effect is closely associated with graft-versus host disease (GVHD), which remains one of the main causes of transplantation-related mortality (TRM) after HSCT [1,2]. Acute GVHD (aGVHD) is staged and graded according to symptom severity in 3 main target organs: skin, liver, and the gastrointestinal tract [3,4], and first-line treatment remains high-dose corticosteroids, typically starting at 1 to 2 mg/kg/day [5]. Unfortunately, up to 50% of patients respond inadequately to this treatment, resulting in a high mortality rate, and there is no consensus about the optimal second- and third-line treatments [6,7].

Although many risk factors for the development of aGVHD have been identified, less is known about risk factors predicting initial treatment failure and steroid-refractory disease in patients with aGVHD. aGVHD grade at onset, organ involvement, maximum aGVHD grade, and time to initial

Financial disclosure: See Acknowledgments on page 606.
* Correspondence and reprint requests: Lia Minculescu, MD, Department of Clinical Immunology, Copenhagen University Hospital, Rigshospitalet, Ole Maaloees Vej 26, Copenhagen N 2200, Denmark.
E-mail address: lia.minculescu@regionh.dk (L. Minculescu).

https://doi.org/10.1016/j.bbmt.2017.10.025

treatment response have shown some association with patient outcomes and survival [7-9]. Because the severity of symptoms at aGVHD diagnosis does not always accurately define risk, the search for prognostic biomarkers is ongoing [10]. Decreased serum albumin level and lymphocytopenia at diagnosis have been associated with initial treatment failure and decreased survival in patients with aGVHD [11,12]. Fecal biomarkers have similarly been shown to predict steroid response in patients with gastrointestinal aGVHD [13]. There is increasing evidence that coexisting inflammation not only aggravates aGVHD, but also contributes to the initial pathogenesis [14,15]. The underlying disease, together with the pre-HSCT conditioning regimen, creates an environment of inflammation and increased activation of antigen-presenting cells in target organs. This leads to activation of alloreactive donor T cells and further release of proinflammatory cytokines, resulting in an amplified and self-perpetuating inflammation cascade that causes tissue damage to target organs [16]. Logically, many of the investigated biomarkers reflect specific proinflammatory cytokines or pathways in this inflammatory process [10,17,18].

The acute-phase protein C-reactive protein (CRP) is a widely used systemic inflammatory marker produced by hepatocytes downstream of IL-6 during an acute inflammatory response [19]. CRP has previously been investigated in the setting of HSCT [20-26]. It has been shown that patterns of CRP levels early after transplantation may correlate with both TRM [24] and relapse [22], and that pre-engraftment CRP level may predict the occurrence of GVHD in HSCT recipients [20]. Others have reported no increases in plasma levels of IL-6 and CRP in the course of GVHD, however [23,25]. To our knowledge, no previous studies have evaluated CRP levels at the time of aGVHD diagnosis in relation to the initial treatment response. The aim of the present study was to assess whether the plasma CRP concentration at the onset of aGVHD is predictive of steroid-refractory aGVHD and TRM in a retrospective cohort of patients with grade II-IV aGVHD.

PATIENTS AND METHODS

Patients

A total of 511 patients underwent HSCT at the Bone Marrow Transplantation Unit, Department of Hematology, Copenhagen University Hospital, Rigshospitalet between January 2010 and December 2015. Patients undergoing transplantation with umbilical cord blood, treated with donor lymphocyte infusions, or undergoing more than 1 transplantation were excluded, leaving 464 patients for evaluation. Three of these patients did not have data on CRP level and differentiated WBC available for analysis at time of aGVHD diagnosis. Patient and transplantation characteristics for the remaining 461 patients included in this study are presented in Table 1. The median patient age was 53 years (range, 16-75 years), and the median duration of follow-up was 1340 days (range, 311-2480 days). Signed informed consent was obtained from each patient or a legal guardian allowing analysis of clinical and laboratory data for research in advance.

Table 1
Patient and Transplantation Characteristics

Characteristic	Value
Patients, n	461
Follow-up time, d, median, range	1340 (311-2480)
Age, yr, median, range	53 (16-75)
Disease, n	
Acute myelogenous leukemia	173
Myelodysplastic syndrome	111
Acute lymphoblastic leukemia	58
Non-Hodgkin lymphoma	37
Chronic lymphocytic leukemia	27
Chronic myelogenous leukemia	18
Severe aplastic anemia	15
Other	22
Risk score*, n	
Early	262
Intermediate	184
Late	15
Donor type, n	
Sibling	115
Other matched related	1
Matched unrelated	307
Mismatched unrelated	38
Patient-donor sex match, n	
Female-female	100
Female-male	71
Male-male	202
Male-female	88
Stem cell source, n	
Bone marrow	118
PBSCs	343
Conditioning regimen, n	
Myeloablative	201
Reduced intensity	7
Nonmyeloablative	253
Conditioning regimen, n	
Cyclophosphamide-TBI	87
Cyclophosphamide-busulfan	15
Etophophos-TBI	49
Fludarabine-TBI	250
Fludarabine-treosulfan	32
Cyclophosphamide-ATG +/- TBI	15
Other	13
aGVHD, n	204
aGVHD grade I, n	56
aGVHD grade II, n	102
aGVHD grade III, n	34
aGVHD grade IV, n	12
Chronic GVHD, n	227

* Risk score from the European Group for Blood and Marrow Transplantation.

Donors and Stem Cell Sources

One hundred and fifteen patients had an HLA-identical sibling donor, and 1 patient had a 10/10 HLA allele-identical other related donor (mother). Three hundred and seven patients had a 10/10 or 9/10 HLA allele-matched unrelated donor, and 35 patients had an unrelated donor with an antigen mismatch. Three patients had an unrelated donor with more than 1 antigen mismatch. The stem cell source was bone marrow (BM) or granulocyte colony-stimulating factor-stimulated unmanipulated peripheral blood stem cells (PBSCs).

Conditioning Regimen

Myeloablative regimens were cyclophosphamide 120 mg/kg plus 12 Gy of total body irradiation (TBI) or busulfan (Busilvex) 12.2 mg/kg in myeloid diseases; cyclophosphamide was replaced with etoposide phosphate (Etophophos) 1800 mg/m^2 in lymphoid diseases. Doses of cyclophosphamide and Busilvex were calculated using an adjusted ideal body weight in patients with a body mass index >27.5. Nonmyeloablative conditioning was provided with fludarabine 90 mg/m^2 plus 2 Gy TBI, increased to 4 Gy in patients not previously treated with chemotherapy. Patients with high-risk myelodysplastic syndrome were conditioned with fludarabine 150 mg/kg plus treosulfan 42 mg/kg. The conditioning regimen for patients with severe aplastic anemia was cyclophosphamide 200 mg/kg plus 2 Gy TBI and thymoglobulin 6.75 mg/kg in those with an unrelated donor.

GVHD Prophylaxis

Patients receiving myeloablative conditioning were given oral cyclosporine 6.25 mg/kg twice daily starting on day -1 combined with a short course of i.v. methotrexate on days +1 (15 mg/m^2), +3, +6, and +11 (10 mg/m^2). Cyclosporine was tapered to stop on day +180, unless GVHD was present. Tacrolimus was administered to the patients receiving the fludarabine/treosulfan regimen. Patients receiving nonmyeloablative conditioning were given oral tacrolimus .06 mg/kg twice daily starting on day -3 along with mycophenolate mofetil 15 mg/kg twice daily from day 0 to day +27 in recipients of a related donor transplant. In recipients of an unrelated transplant, oral tacrolimus .06 mg/kg twice daily starting on day -3 was combined with oral mycophenolate mofetil 15 mg/kg three times daily from day 0 to day +30, twice daily to day +40, and then tapered to stop on day +96 in the absence of GVHD. In the absence of GVHD, tacrolimus was tapered starting on day +56 to zero by day +180 in recipients of related nonmyeloablative

transplants, and from day +100 to zero by day +180 in recipients of unrelated nonmyeloablative transplants.

GVHD Diagnosis and Treatment

aGVHD and chronic GVHD were diagnosed and graded based on clinical symptoms and biopsy analysis according to the modified Glucksberg-Seattle criteria [3,4]. aGVHD grade II-IV was treated with methylprednisolone 2 mg/kg. Patients showing disease progress within 3 days or inadequate response (ie, no response) within 1 week on this treatment were started on second-line treatment consisting of TNF-α antibody (infliximab 10 mg/kg) and occasionally extracorporeal photopheresis (ECP). Patients treated with infliximab were categorized as having steroid-refractory disease in this study. Chronic GVHD was treated with prednisolone 1 mg/kg, supplemented with calcineurin inhibitors, mycophenolate mofetil, sirolimus, or ECP at the discretion of the treating physician.

Overall Survival and Transplant Related Mortality

Overall survival (OS) was defined as the probability of survival from the time of HSCT to time of death or last follow-up. Surviving patients were censored at last follow-up, and only death was considered an event. TRM was defined as death from causes other than relapse.

Laboratory Samples

In our institution, plasma CRP concentrations, together with differentiated WBC, are measured routinely after HSCT and consistently in patients under observation for the development of aGVHD. CRP concentration in plasma was measured using the CRPL3 turbidimetric assay (Modular P chemistry analyzer; Hitachi, Tokyo, Japan) (normal range, 0-10 mg/L). In this study, all patients had a CRP/WBC measurement on the day of aGVHD diagnosis or 1 day before.

Statistical Analyses and Endpoints

The primary aim of this study was to evaluate CRP levels at the time of aGVHD diagnosis in patients with aGVHD grade II-IV in correlation with the development of steroid-refractory disease and subsequent TRM and OS. Secondary aims were to evaluate WBC and subgroups for the same outcomes. Logistic regression with log-transformed continuous variables was used to assess correlation. Differences between categorical and continuous variables were determined by the χ^2 test and Student's t test, respectively. Differences among group means (more than 2 groups) were analyzed by one-way analysis of variance. Estimated OS was determined by univariate analysis using the Kaplan-Meier method and the log-rank test. Cumulative incidence rates of TRM, GVHD, and relapse with competing events were compared using Gray's test. All P values were 2-sided and considered statistically significant at <.05. CRP level in relation to the occurrence of steroid-refractory aGVHD was analyzed as a continuous variable in logistic regression and as a dichotomized, categorical variable in survival and cumulative incidence analyses. In the logistic regression analysis, the log2 transformation of CRP level was performed to fit the statistical model for logistic regression. Thus, interpretation of the odds ratio (OR) is the estimated increase in probability of developing steroid-refractory aGVHD by each doubling of any arbitrary CRP level. CRP was used as a continuous variable in logistic regression analysis to obtain a statistically robust and direct correlation with the development of steroid-refractory disease. In the Kaplan-Meier analysis for OS and the Gray's test for cumulative incidence, CRP levels were dichotomized at the normal cutoff of 10 mg/L. Statistical analyses were performed using SPSS version 22 (SPSS, Chicago, IL), SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC), and R version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria) combined with the EZR platform [27].

RESULTS

Among the 461 patients, 300 (65%) were alive after a median follow-up of 1340 days (range, 311-2480 days). Seventy-four patients (16%) died from relapse, and 87 (19%) died from TRM. aGVHD was diagnosed in 203 patients (44%), and 227 patients (49%) experienced some degree of chronic GVHD during the observation period (Table 1).

aGVHD Grade II-IV

A total of 148 patients (32%) developed grade II-IV aGVHD, 28 (19%) of whom developed steroid-refractory disease. No patients with initial grade I aGVHD developed steroid-refractory disease. Table 2 shows associations between baseline factors and the development of grade II-IV aGVHD and steroid-refractory aGVHD in all 461 patients. Age and graft source showed statistically significant associations with the occurrence of grade II-IV aGVHD, and the use of high-intensity TBI (12 Gy) was highly significantly associated with the development of grade II-IV aGVHD. In multivariate analysis including all of the factors listed in Table 2, only the use of high-intensity TBI showed a statistically significant association with the development of grade II-IV aGVHD (data not shown). None of the factors investigated showed a statistically significant association with the development of steroid-refractory aGVHD, although there was a tendency toward an association between high-intensity TBI and an increased incidence of steroid-refractory disease in the univariate analyses (Table 2).

The distribution of grade and organ involvement in the 148 patients grade II-IV aGVHD patients is shown in Table 3. Of the 28 patients who developed steroid- refractory disease,

Table 2
Baseline Characteristics and Univariate Association with the Development of aGVHD Grade II-IV and Steroid-Refractory aGVHD in All Patients (n = 461)

Characteristic	Patients, n	aGVHD Grade II-IV			Steroid-Refractory aGVHD		
		n (%)	OR (95% CI)	*P* Value	n (%)	OR (95% CI)	*P* Value
Age, yr							.59
≤53	237	86 (36)	Reference	.05	13 (5.5)	Reference	
>53 (median)	224	62(28)	.67 (.45-1.00)		15 (6.7)	1.24 (.58-2.66)	
Conditioning regimen							.48
Myeloablative	201	80 (40)	Reference	.02	14 (7.0)	Reference	
Nonmyeloablative	260	68 (26)	.54 (.36-.80)		14 (5.4)	.76 (.35-1.63)	
TBI							.12
12 Gy	137	67 (49)	Reference	<.00	12 (8.8)	Reference	
Other	324	81 (25)	.35 (.23-.53)	1	16 (4.9)	.54 (.25-1.18)	
Donor							.67
Sibling	116	32 (28)	Reference	.23	8 (6.9)	Reference	
Matched unrelated	345	116 (34)	1.33 (.84-2.12)		20 (5.8)	.83 (.36-1.94)	
Donor/patient sex							.87
Female/male	71	25 (35)	Reference	.54	4 (5.6)	Reference	
Other	390	123 (32)	.85 (.50-1.44)		24(6.2)	1.10 (.37-3.27)	
Allogeneic match							.36
9/10 or 10/10 allele match	422	136 (32)	Reference	.85	27 (6.4)	Reference	
Other	39	12 (31)	.94 (.46-1.90)		1 (2.6)	.39 (.05-2.91)	
Graft source							.94
Bone marrow	118	47 (40)	Reference	.04	7 (5.9)	Reference	
PBSCs	343	101 (29)	.63 (.41-.98)		21 (6.1)	1.03 (-43-2.50)	

Table 3
Disease Characteristics in Patients Developing Grade II-IV aGVHD (n = 148)

Characteristic	Value
Time to aGVHD diagnosis, d, median (range)	34 (8-132)
Grade at diagnosis, n	
Grade II	108
Grade III	40
Maximum grade, n	
Grade II	102
Grade III	34
Grade IV	12
Organ involvement, n	
Skin	88
Skin + gut	23
Skin + liver	5
Gut	28
Gut + liver	2
Liver	2
Verified involvement of multiple organs, n	30
Visceral involvement, n	62

22 (79%) died from TRM, at a median of 33 days (range, 8-384 days) from the first infliximab infusion. aGVHD was the direct cause of death in 16 of these patients, whereas the other 6 patients died from infection or organ failure.

CRP Levels and Steroid-Refractory Disease, TRM, and OS in Patients with Grade II-IV aGVHD

Due to the inclusion criteria, laboratory results, including CRP levels, were available for all patients with grade II-IV aGVHD at the time of diagnosis or the day before. CRP levels at diagnosis were between <1 and 253 mg/L (median, 17 mg/L). CRP levels at diagnosis were significantly higher in patients who developed steroid-refractory disease compared with those who responded to high-dose corticosteroids (OR, 1.50; 95% CI, 1.18-1.93; $P = .001$). Because CRP was analyzed as a log2-transformed, continuous variable in the logistic regression analysis, the OR of 1.50 is interpreted as a 50% increase in the estimated probability of developing steroid-resistant disease by each doubling of any arbitrary CRP level. Patients with grade II-IV aGVHD with high CRP levels at diagnosis had significantly increased TRM compared with those with lower CRP levels: Figure 1 shows this association by patients divided into 2 groups by normal/elevated (>10 mg/L) CRP, with 56 patients in the ≤10 mg/L group and 92 patients in the >10 mg/L group. The increase in TRM translated into a significantly lower OS in these patients (Figure 2).

Steroid-Refractory aGVHD and Other Factors

Table 4 lists baseline factors and possible risk factors at diagnosis evaluated for associations with the development of steroid-refractory aGVHD exclusively in patients with grade II-IV aGVHD. None of the analyzed baseline factors or the time to aGVHD onset showed a significant association with the development of steroid-refractory disease; however, grade at onset (II versus III, given that no patients presented with grade IV at diagnosis) and visceral involvement showed highly significant associations with increased risk of steroid-refractory aGVHD. To evaluate whether CRP levels were predictive of the development of steroid-refractory disease independent of aGVHD grade at diagnosis and visceral involvement, we performed a multivariate analysis with CRP (a log2-transformed continuous variable), grade at diagnosis (II versus III) and visceral involvement (yes versus no) in the 148 patients with grade II-IV aGVHD (Table 5). Although aGVHD grade at diagnosis remained highly statistically significant ($P < .0001$), CRP level now failed to reach statistical significance ($P = .16$), and visceral involvement showed no tendency

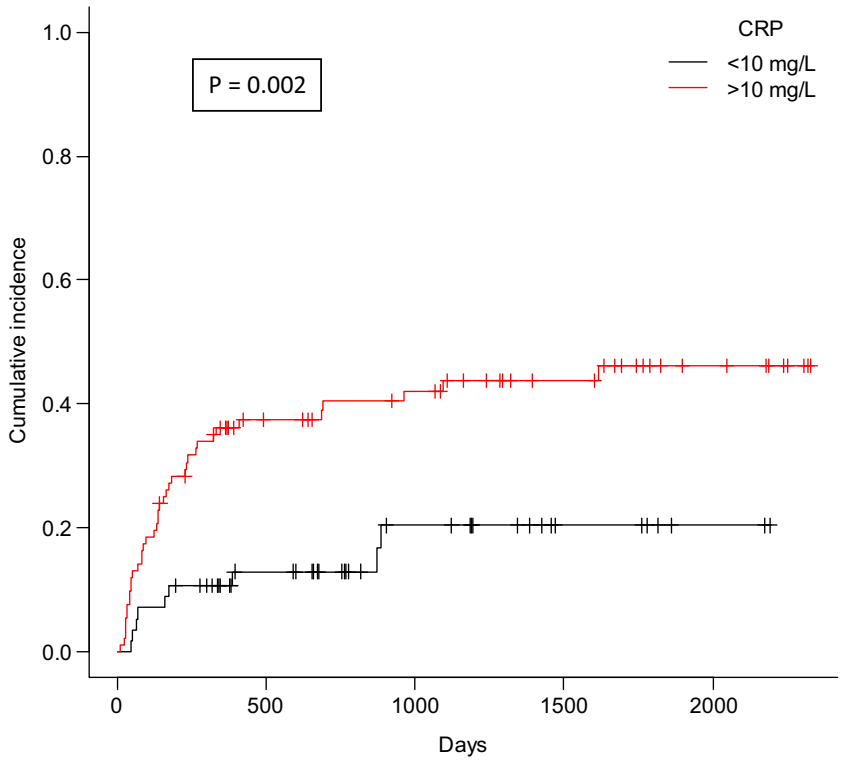

Figure 1. Cumulative incidence of TRM with death from relapse as competing event by CRP levels at diagnosis above and below 10 mg/L in patients with grade II-IV aGVHD, n = 148.

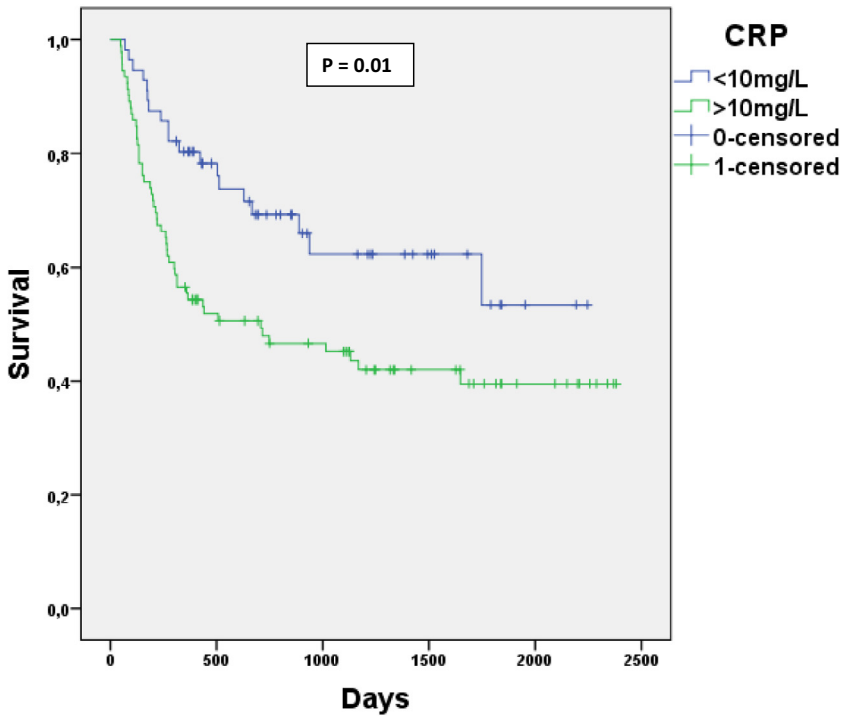

Figure 2. Kaplan Meier estimate of OS by CRP levels >10 mg/L and <10 mg/L at diagnosis in patients with grade II-IV aGVHD (n = 148).

Table 4
Possible Risk Factors Associated with the Development of Steroid-Refractory Disease in Patients with Grade II-IV aGVHD at Baseline and at Diagnosis (n = 148)

Characteristic Baseline	Patients, n	Steroid-Refractory, n (%)	OR (95% CI)	*P* Value
Age, yr				.27
<49	72	11 (15)	.63 (.27-1.45)	
>49 (median)	76	17 (22)	Reference	
Conditioning regimen				.63
Myeloablative	80	14 (18)	Reference	
Nonmyeloablative	68	14 (21)	1.22 (.53-2.79)	
TBI				.78
12 Gy	67	12 (18)	Reference	
Other	81	16 (20)	1.13 (.49-2.59)	
Donor				.32
Sibling	32	8 (25)	1.60 (.63-4.07)	
Matched unrelated	116	20 (17)	Reference	
Donor/patient sex				.68
Female/male	25	4 (16)	Reference	
Other	123	24 (20)	1.27 (.40-4.05)	
Allogeneic match				.35
9/10 or 10/10 allele match	109	27 (25)	2.7 (.34-22.03)	
Other	11	1 (9)	Reference	
Graft source				.40
Bone marrow	47	7 (15)	.67 (.26-1.7)	
PBSCs	101	21 (21)	Reference	
At aGVHD diagnosis				
Days from HSCT to aGVHD, d				.78
<30	67	12 (18)	.89 (.39-2.03)	
>30	81	16 (20)	Reference	
aGVHD grade at diagnosis				<.001
II	108	6 (6)	.05 (.02-.14)	
III	40	22 (55)	Reference	
Visceral involvement in aGVHD				.001
Yes	56	19 (34)	Reference	
No	92	9 (10)	.21 (.09-.51)	
CRP level at diagnosis, g/mL				.05
<10	56	6 (11)	.38 (.14-1.0)	
>10	92	22 (24)	Reference	
Log2 CRP, continuous variable			1.50 (1.18-1.93)	.001

Table 5
Multivariate Analyses of Possible Risk Factors Associated with the Development of Steroid-Refractory Disease in Patients with Grade II-IV and Grade II and III Separately at Diagnosis of a GVHD

aGVHD Grade	OR (95% CI)	*P* value
II-IV (n = 148)		
CRP	1.19 (.93-1.53)	.16
Grade at aGVHD diagnosis	.07 (.02-0.22)	<.0001
Visceral involvement	.96 (.29-3.17)	.94
II (n = 108)		
CRP	2.23 (1.16-4.25)	.02
Visceral involvement	.55 (.08-3.61)	.55
III (n = 40)		
CRP	.98 (.72-1.33)	.89
Visceral involvement	1.00 (.22-4.66)	1.00

toward any statistical association ($P = .94$). Because there was still a tendency toward a higher incidence of steroid-refractory disease with increasing CRP levels, we analyzed grade II and grade III aGVHD separately in multivariate analyses together with visceral involvement (Table 5). In patients with grade II aGVHD (n = 108), CRP level was associated with an increased risk of steroid-refractory disease ($P = .02$), whereas this association was not found in those with grade III aGVHD (n = 40) ($P = .89$). Visceral involvement was not associated with steroid-refractory disease in any group.

Total WBC, lymphocyte, and neutrophil counts were analyzed as continuous variables, and no association with the development of steroid-refractory aGVHD was found (data not shown).

CRP Levels and Steroid-Refractory aGVHD in Different Organ Systems

The mean CRP levels at diagnosis for liver, gastrointestinal, and skin aGVHD were 104 mg/L (range, 9-195 mg/L), 60 mg/L (range, 0-253 mg/L), and 36 mg/L (range, 0-223 mg/L), respectively. One-way analysis of variance confirmed a statistically significant difference in mean values (data not shown). We then analyzed skin aGVHD (n = 116) and gastrointestinal aGVHD (n = 53) separately in univariate and multivariate analyses as described above, and found the same results as for all patients with grade II-IV aGVHD. CRP level at diagnosis was significantly associated with an increased risk of developing steroid-refractory disease in univariate analysis, but the association was no longer significant in multivariate analyses with aGVHD grade at diagnosis included. The associations were similar for skin and gastrointestinal aGVHD, data not shown. Given that only 9 patients had registered liver aGVHD at diagnosis, a separate analysis was not performed for these patients.

DISCUSSION

In this study, we performed a retrospective analysis on the potential for CRP level at diagnosis as a biomarker for the development steroid-refractory aGVHD after HSCT. In our study population of 461 patients who underwent HSCT for a variety of malignant and nonmalignant diseases, the incidence of steroid-refractory disease in patients with grade II-IV aGVHD was 19%. Mortality was 79% in patients who did not respond to high-dose steroid therapy. In patients with grade II-IV aGVHD, increased CRP level at diagnosis predicted the development of steroid-refractory disease. This finding is consistent with the understanding of CRP as a marker of inflammation. CRP is produced downstream of IL-6, which, together with TNF-α and IFN-γ, are key players in the proinflammatory cytokine cascade of aGVHD pathogenesis [1,16]. Several preclinical and clinical studies have reported the key role of IL-6 in driving GVHD [17,28,29]. Chen et al. [28] found that blockade of IL-6 signaling attenuated the severity of GVHD in mice, and McDonald et al. [17] showed that IL-6 level at the diagnosis of aGVHD predicted the development of high-grade (III-IV) aGVHD in a prospective multicohort study of patients with gastrointestinal aGVHD.

Other factors associated with an increased risk of steroid-refractory disease in patients with grade II-IV aGVHD are high-grade disease at diagnosis and visceral involvement. Other studies have found associations with these clinical factors [7,8]; however, recent studies have shown that algorithms containing biomarkers strengthen the clinical prediction of failure of first-line treatment [10,18,30]. In our patient cohort, the impact of aGVHD grade at diagnosis remained highly statistically associated with steroid-refractory disease in the multivariate analyses; however, the association with visceral involvement disappears in multivariate analyses of all patients with grade II-IV aGVHD and the separate analyses of those with grade II and III at onset. CRP level lost statistical significance when aGVHD grade at diagnosis and visceral involvement were included in the analysis of all patients with grade II-IV aGVHD, making grade at diagnosis a highly potent predictor of initial treatment response. However, the majority (73%) of patients with grade II-IV aGVHD in our cohort presented with grade II at diagnosis, and higher CRP level remained significantly associated with an increased risk of steroid-refractory disease in a subgroup analysis of this patient group (n = 108). The reason for the lack of any association with steroid-refractory aGVHD in patients presenting with grade III aGVHD at diagnosis might be the relatively low number of patients (n = 40). In our cohort, patients with registered liver aGVHD at diagnosis had significantly higher CRP levels compared with patients without liver involvement. This might be consistent with the assumption that hepatic inflammation raises CRP, but should be verified in a larger patient cohort. The statistically significant association between CRP level and steroid-refractory disease was similar in patients with skin and gastrointestinal aGVHD analyzed separately, and the association lost statistical significance when aGVHD grade at diagnosis was included in multivariate analyses. The association between liver aGVHD and CRP level was not analyzed separately because of the low number of patients (n = 9). Whether the possible predictive value of CRP level on the development of steroid-refractory disease differs in different organ systems with different initial grades merit investigation in future prospective studies with larger patient numbers.

CRP level was associated with an increased risk of steroid-refractory disease in patients with grade II-IV aGVHD, and because aGVHD is one of the major causes of TRM, we evaluated the potential impact of CRP level at diagnosis on TRM in the 148 patients with grade II-IV aGVHD. We found that CRP level at diagnosis predicted an increased risk of death from TRM, which translated into decreased OS. A major confounding factor could be the occurrence of infection at the time of aGVHD diagnosis, with a possible impact of CRP levels. A recent study confirmed the mutual impact of aGVHD and infections on each other, not only with the increased risk of infections due to immunosuppression from aGVHD treatment, but also with reported increased risk of infection before the onset of aGVHD [31]. Unfortunately, data on infections at the time of aGVHD diagnosis were limited in this retrospective study, and because we could not adjust for this factor, the impact of concurrent infections on TRM cannot be

excluded. Therefore, TRM and OS were analyzed only in a univariate manner in this study, and so the main finding remains the direct association between CRP level and the risk of steroid-refractory aGVHD. Detailed data on possible infections together with parallel analyses of other inflammatory markers, such as procalcitonin levels, may be useful in prospective studies of the impact of CRP level in patients with aGVHD [23]. Moreover, in this study, only the CRP level at the time of aGVHD diagnosis was reported and analyzed in relation to the development of steroid-refractory disease. In prospective studies including information on the possible competing influence of concurrent infections on CRP level, the kinetics of CRP levels through the course of aGVHD treatment might further elucidate the clinical value of CRP level as a predictive marker of treatment response, together with the initial level at diagnosis.

Several other factors, including conditioning regimen, aplasia, mucositis and engraftment syndrome, could affect CRP levels during the course of HSCT [26]. Conditioning regimens have been shown to increase CRP levels to various degrees, with antithymocyte globulin-containing regimens the most influential [32,33]. These factors seem to affect CRP levels primarily during the first 1 to 2 weeks after HSCT, and because the median time to diagnosis of grade II-IV aGVHD in our patient cohort was 33 days, we believe that the impact of these additional factors was less dominant. However, further studies of CRP levels in a larger context at the time of diagnosis of aGVHD are warranted to verify our findings.

Our univariate analyses of baseline risk factors and the occurrence of grade II-IV aGVHD in our patient cohort revealed somewhat unexpected associations with young age and graft source. The association between the use of bone marrow and increased occurrence of grade II-IV aGVHD may be explained by the increased use of high- intensity TBI in patients receiving this graft source; the loss of statistical significance between bone marrow and PBSCs in multivariate analyses supports this idea. Surprisingly, we found no association between such otherwise known risk factors as allogeneic mismatch, the use of unrelated donors, or increased intensity of the conditioning regimen [34] in univariate analyses. The strongest association with grade II-IV aGVHD was with high-dose TBI as described previously [34]. This supports the understanding, that local inflammation and endothelial tissue damage caused by irradiation is an initial phase in the pathogenesis of aGVHD [1,16]. No statistical associations were observed between baseline factors and the occurrence of steroid-refractory aGVHD in either the patients with grade II-IV aGVHD or the entire patient cohort. This may have been related to the low number of incidences (28 in 461 patients), but also confirms the need for prognostic markers at the time of clinical aGVHD manifestation.

In this study, steroid-refractory aGVHD was categorized as treatment failure within 3 days or as inadequate response to high-dose steroid treatment within 1 week. In prospective studies, standardized treatment response assessments at defined time points may contribute to the evaluation of the prognostic value of CRP level at diagnosis.

To demonstrate a direct and statistically robust correlation between CRP level at aGVHD diagnosis and the development of steroid-resistant disease, we included CRP level as a continuous variable in the univariate and multivariate logistic regression analyses. In the survival analyses, we chose to dichotomize CRP levels by the normal cutoff of 10 mg/L, because more than one-third of patients with grade II-IV aGVHD actually had a CRP level within the normal range at diagnosis. Of course, alternative cutoffs or approaches could be considered in future studies.

Contrary to other studies [11], we did not find any association between lymphocyte count and the development of steroid-refractory aGVHD; lymphocyte level was analyzed both as a continuous variable as well as by previously described cutoff of 100 cells/μL. Albumin is another standard serum marker analyzed in the course of HSCT, which has been demonstrated to be potentially prognostic for the development of treatment failure in aGVHD [11,12]. Unfortunately, laboratory albumin results were available in only a small portion of our patient cohort and thus was not analyzed.

Other biomarkers identified as prognostic for the severity of aGVHD include ST2, TIM3, IL-6, TNFR1, and REG3α, all of which are included in the regulation of inflammatory processes in relation to tissue damage [10,17]. The 2015 multicenter study by Levine et al. [10] identified the validated plasma biomarkers ST2, TNF, and REG3α as valuable in predicting 6-month nonrelapse mortality after the onset of aGVHD independent of clinical grading systems, and suggested a prognostic aGVHD score based on biomarkers. In gastrointestinal aGVHD, the fecal biomarkers calprotectin and alpha-1 antitrypsin have similarly been reported as predictive of treatment response, along with grade at onset [13]. The role of CRP in the context of these specific plasma and/or fecal biomarkers is not known, but because plasma CRP analysis is a widely used and easy accessible test, a parallel investigation of CRP in future studies on specific prognostic biomarkers could be considered.

CONCLUSION

The poor outcomes in patients developing steroid-refractory aGVHD in this study confirms the need for improved prognostic biomarkers for predicting the response to initial treatment. We identified elevated CRP level at the time of aGVHD diagnosis as significantly associated with the development of steroid-refractory disease in patients with grade II-IV aGVHD. Closer monitoring with potentially earlier interventions with second-line treatment in patients at increased risk could improve treatment outcomes. Monitoring of CRP levels along with clinical risk factors and additional biomarkers of inflammation could be included in risk assessment at the time of aGVHD diagnosis. Prospective studies to further clarify the role of CRP level as a prognostic biomarker of steroid-refractory aGVHD after HSCT are warranted.

ACKNOWLEDGMENTS

Financial disclosure: This work was supported by unrestricted grants from the Danish Cancer Society, (R90-A5858) the Novo Nordisk Foundation (Grant 15OC0014158), and the Danish National Research Foundation (Grant 126).

Conflict of interest statement: There are no conflicts of interest to report.

REFERENCES

1. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. 2009;373:1550-1561.
2. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. *Blood*. 2014;124:363-373.
3. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. *Transplantation*. 1974;18:295-304.
4. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15:825-828.

5. Martin PJ, Rizzo JD, Wingard JR, et al. First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2012;18:1150-1163.
6. Minculescu L, Madsen HO, Sengeløv H. T-cell chimerism is valuable in predicting early mortality in steroid-resistant acute graft-versus-host disease after myeloablative allogeneic cell transplantation. *Acta Haematol*. 2014;132:187-192.
7. Westin JR, Saliba RM, De Lima M, et al. Steroid-refractory acute GVHD: predictors and outcomes. *Adv Hematol*. 2011;2011:601953.
8. Levine JE, Logan B, Wu J, et al. Graft-versus-host disease treatment: predictors of survival. *Biol Blood Marrow Transplant*. 2010;16:1693-1699.
9. McDonald GB, Tabellini L, Storer BE, et al. Predictive value of clinical findings and plasma biomarkers after fourteen days of prednisone treatment for acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2017;23:1257-1263.
10. Levine JE, Braun TM, Harris AC, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. *Lancet Haematol*. 2015;2:e21-e29.
11. Lee KH, Choi SJ, Lee JH, et al. Prognostic factors identifiable at the time of onset of acute graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Haematologica*. 2005;90:939-948.
12. Rezvani AR, Storer BE, Storb RF, et al. Decreased serum albumin as a biomarker for severe acute graft-versus-host disease after reduced-intensity allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2011;17:1594-1601.
13. Rodriguez-Otero P, Porcher R, Peffault de Latour R, et al. Fecal calprotectin and alpha-1 antitrypsin predict severity and response to corticosteroids in gastrointestinal graft-versus-host disease. *Blood*. 2012;119:5909-5917.
14. Choi SW, Levine JE, Ferrara JL. Pathogenesis and management of graft-versus-host disease. *Immunol Allergy Clin North Am*. 2010;30:75-101.
15. Penack O, Holler E, van den Brink MR. Graft-versus-host disease: regulation by microbe-associated molecules and innate immune receptors. *Blood*. 2010;115:1865-1872.
16. Magenau J, Runaas L, Reddy P. Advances in understanding the pathogenesis of graft-versus-host disease. *Br J Haematol*. 2016;173:190-205.
17. McDonald GB, Tabellini L, Storer BE, Lawler RL, Martin PJ, Hansen JA. Plasma biomarkers of acute GVHD and nonrelapse mortality: predictive value of measurements before GVHD onset and treatment. *Blood*. 2015;126:113-120.
18. Paczesny S, Krijanovski OI, Braun TM, et al. A biomarker panel for acute graft-versus-host disease. *Blood*. 2009;113:273-278.
19. Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem*. 2004;279:48487-48490.
20. Fuji S, Kim SW, Fukuda T, et al. Preengraftment serum C-reactive protein (CRP) value may predict acute graft-versus-host disease and nonrelapse mortality after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2008;14:510-517.
21. Jordan KK, Christensen IJ, Heilmann C. Pretransplant C-reactive protein as a prognostic marker in allogeneic stem cell transplantation. *Scand J Immunol*. 2014;79:206-213.
22. Min CK, Kim SY, Eom KS, et al. Patterns of C-reactive protein release following allogeneic stem cell transplantation are correlated with leukemic relapse. *Bone Marrow Transplant*. 2006;37:493-498.
23. Pihusch M, Pihusch R, Fraunberger P, et al. Evaluation of C-reactive protein, interleukin-6, and procalcitonin levels in allogeneic hematopoietic stem cell recipients. *Eur J Haematol*. 2006;76:93-101.
24. Schots R, Van Riet I, Othman TB, et al. An early increase in serum levels of C-reactive protein is an independent risk factor for the occurrence of major complications and 100-day transplant-related mortality after allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 2002;30:441-446.
25. Schwaighofer H, Herold M, Schwarz T, et al. Serum levels of interleukin 6, interleukin 8, and C-reactive protein after human allogeneic bone marrow transplantation. *Transplantation*. 1994;58:430-436.
26. Sjøqvist C, Snarski E. Inflammatory markers in patients after hematopoietic stem cell transplantation. *Arch Immunol Ther Exp (Warsz)*. 2013;61:301-307.
27. Kanda Y. Investigation of the freely available easy-to-use software "EZR" for medical statistics. *Bone Marrow Transplant*. 2013;48:452-458.
28. Chen X, Das R, Komorowski R, et al. Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood*. 2009;114:891-900.
29. Symington FW, Symington BE, Liu PY, Viguet H, Santhanam U, Sehgal PB. The relationship of serum IL-6 levels to acute graft-versus-host disease and hepatorenal disease after human bone marrow transplantation. *Transplantation*. 1992;54:457-462.
30. Vander Lugt MT, Braun TM, Hanash S, et al. ST2 as a marker for risk of therapy-resistant graft-versus-host disease and death. *N Engl J Med*. 2013;369:529-539.
31. Miller HK, Braun TM, Stillwell T, et al. Infectious risk after allogeneic hematopoietic cell transplantation complicated by acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2017;23:522-528.
32. Brodska H, Drabek T, Malickova K, et al. Marked increase of procalcitonin after the administration of anti-thymocyte globulin in patients before hematopoietic stem cell transplantation does not indicate sepsis: a prospective study. *Crit Care*. 2009;13:R37.
33. van der Velden WJ, Herbers AH, Feuth T, Schaap NP, Donnelly JP, Blijlevens NM. Intestinal damage determines the inflammatory response and early complications in patients receiving conditioning for a stem cell transplantation. *PLoS One*. 2010;5:e15156.
34. Flowers ME, Inamoto Y, Carpenter PA, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood*. 2011;117:3214-3219.